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Biochimie. Author manuscript; available in PMC 2010 January 1.

Published in final edited form as:
Biochimie. 2009 January ; 91(1): 76–85. doi:10.1016/j.biochi.2008.04.008.

Influence of bovine lactoferrin on expression of presentation molecules on BCG-infected bone marrow derived macrophages

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Abstract

The current vaccine for tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is an attenuated strain of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). BCG has proven to be effective in children, however, efficacy wanes in adulthood. Lactoferrin, a natural protein with immunomodulatory properties, is a potential adjuvant candidate to enhance efficacy of BCG. These studies define bovine lactoferrin as an enhancer of the BCG vaccine, functioning in part by modulating macrophage ability to present antigen and stimulate T-cells. BCG-infected bone marrow derived macrophages (BMMs) cultured with bovine lactoferrin increased the number of MHC II$^+$ expressing cells. Addition of IFN-γ and lactoferrin to BCG-infected BMMs enhanced MHC II expressiona dna increased the ratio of CD86/CD80. Lactoferrin treated BCG-infected BMMs were able to stimulate an increase in IFN-γ production from presensitized CD3$^+$ splenocytes. Together, these results demonstrate that bovine lactoferrin is capable of modulating BCG-infected macrophages to enhance T-cell stimulation through increased surface expression of antigen presentation and co-stimulatory molecules, which potentially explains the observed *in vivo* bovine lactoferrin enhancement of BCG vaccine efficacy to protect against virulent MTB infection.

Keywords

Lactoferrin; BCG; Vaccine; Adjuvant; Tuberculosis

1. Introduction

It is estimated that Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), currently infects one-third of the world's population and causes nearly 1.7 million deaths per year [1-3]. The existing vaccine is an attenuated strain of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), which, unfortunately, has varying levels of reported efficacy (0–80%). While many novel TB vaccines have been developed [4,5], few have demonstrated protective efficacy that can surpass BCG, which remains the only vaccine approved for human use. Previous research indicates that addition of adjuvant components to the BCG vaccine significantly enhances immune responses to promote host protection against subsequent

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challenge with virulent MTB [6-9]. One such reported component is lactoferrin, an iron binding protein found primarily in mucosal secretions and secondary granules of neutrophils [10].

Lactoferrin possesses multiple biological functions, including bactericidal and bacteriostatic activities [11-15], anti-and pro-inflammatory responses [16], promotion of B- and T-cell maturation [17,18], and enhancement of the delayed type hypersensitivity (DTH) to defined antigens [19-21]. Mice immunized with BCG in the presence of bovine lactoferrin demonstrated increased host protection post-challenge with virulent MTB, as observed by decreasing organ bacterial load and pulmonary disease granulomatous pathology. The BCG/lactoferrin immunized mice displayed increased presence of lymphocytes in granulomas and elevated production of IFN-γ from splenocytes reactive to BCG antigens [22,23]. Overall responses indicated that mice immunized with BCG admixed with bovine lactoferrin developed an enhanced mycobacterial antigen-specific T-cell response, suggesting that facilitation of efficacy of the BCG vaccine is through promotion of development of T-cell helper type 1 (T_H1) immunity.

The T_H1 phenotypic response is, in part, regulated by the presence of IL-12 produced primarily by macrophages and dendritic cells. The IL-12 assists in directed development of naïve $CD4^+$ T-cells towards the T_H1 subtype [24-26]. In addition, IL-12 functions as a co-stimulator for maximizing production of IFN-γ from T_H1 cells and activates IFN-γ production from memory T-cells [27,28]. Lactoferrin has the potential to promote development of both T_H1 and T_H2 immune responses, depending on experimental conditions [29,30]. Towards its utility as an adjuvant, *in vivo* and *in vitro* studies indicate that lactoferrin can increase relative production of IL-12 while decreasing IL-10, a negative regulator of IL-12 [19,31-33]. Of critical importance, bovine lactoferrin added to murine macrophages infected with BCG enhanced the production of IL-12 relative to amounts of IL-10 [34].

Antigenic peptides presented by macrophages via the major histocompatibility complex molecules (MHC) I and II, along with co-stimulatory molecules such as CD80, CD86, and CD40, are essential in the T-cell activation process [35-37]. Recently, lactoferrin was shown to affect surface expression of CD40 on murine macrophages [38], suggesting that lactoferrin may modulate macrophage antigen presentation events to defined antigens. These studies will investigate the hypothesis that lactoferrin is capable of affecting surface expression of molecules on BMMs that are involved in antigen presentation (MHC II, CD80, and CD86), leading to enhanced macrophage stimulation of presensitized T-cell populations. Specifically, these experiments will address the mechanism of bovine lactoferrin to function as an adjuvant component to enhance efficacy of the BCG vaccine, promoting generation of specific immune responses, *in vivo*, and activation of existing specific responses that could assist in host protection against challenge with virulent MTB.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (6 weeks, Jackson Laboratories, Bar Harbor, ME) of 20–25 g initial body weight were used for *in vitro* macrophage development, and as a source of splenocytes. All in vivo experiments were conducted under approved guidelines of the animal welfare ethics committee at the University of Texas, Health Science Center at Houston, protocol HSCAWC-05-060.

2.2. Lactoferrin and BCG

Low endotoxin bovine milk lactoferrin (<0.2 EU/mg, <20% iron saturated, >95% purity) was provided by PharmaReview Corporation (Houston, TX). Endotoxin level was evaluated using

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the Limulus Amebocyte Lysate (BioWhittaker) according to the manufacturer's instructions. *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), Pasteur strain (TMC 1011, ATCC, Manassas, VA) was grown in Dubos base (without addition of glycerol) with 10% supplement (5% BSA and 7.5% dextrose in saline) on an orbital shaker at 37 °C for 2 weeks before use. BCG concentration was estimated using McFarland standards (Sigma) and confirmed by plating dilutions onto 7H11 agar plates (Remel, Lenesa, KS). Plates were incubated at 37 °C for 3–4 weeks prior to enumeration of colonies.

2.3. Derivation and treatment of bone marrow derived macrophages

Bone marrow derived macrophages (BMMs) were differentiated as previously described [39]. Briefly, cells were isolated from C57BL/6 mice (6 weeks, Jackson Laboratories, Bar Harbor, ME) by flushing the femur with McCoy's medium supplemented with 100 μg/mL penicillin G (Sigma) and 50 μg/mL gentamycin sulfate (Sigma). Collected cells were treated with ACK buffer (Cambrex Bio Sciences, East Rutherford, NJ) and resulting cells were differentiated for 7 days at 1×10^6 cells/mL in McCoy's medium, supplemented with 2.2 g/L sodium bicarbonate, 10% FBS, and GM-CSF (10 ng/mL) (Cell Sciences, Canton, MA). At day 7, non-adherent cells were removed and adherent cells (estimated at 5×10^5 cells/well) rested overnight in DMEM complete medium (Dulbecco's modified Eagle's medium (Sigma) with 50 mg/L HEPES (Sigma), 50 mg/L L-arginine (Sigma), 2.2 g/L sodium bicarbonate (Sigma)) supplemented with 10% FBS (Sigma) at 37 °C with 5% CO_2. The adherent cell population was >95% F4/80$^+$ as determined by flow cytometric analysis. Rested macrophages were infected with BCG (MOI 1:1 or 10:1) with or without lactoferrin (100 μg/mL) for 72 h, supernatants were collected and stored at −20 °C for analysis by ELISA, and macrophages were isolated for surface marker staining and analyzed by fluorescence-activated cell sorting (FACS). Comparisons were made to control non-infected cells. To examine macrophage responses to IFN-γ stimulation, macrophages at 72 h post-infection were washed with 1× PBS and cultured with 10 ng/mL mouse recombinant IFN-γ (Cell Sciences). Supernatants and cells were collected at 72 h post-IFN-γ stimulation.

2.4. Macrophage stimulation of presensitized T-cells

Splenocytes from naïve and BCG (1×10^7 CFU/mouse) immunized mice were isolated as previously described [40]. CD3$^+$ T-cells were purified by passing ACK treated splenocytes through a CD3$^+$ enrichment column (R&D Systems, Minneapolis, MN), yielding a minimum of 90% CD3$^+$ cells as determined by FACS analysis. CD4$^+$ and CD8$^+$ T-cells were purified from splenocytes, without ACK buffer treatment, resuspended in 2 mL of Macs buffer (0.5% BSA and 2 mM EDTA in 1× PBS), and incubated with CD4 or CD8 MicroBeads (Miltenyi Biotec) at 4 °C for 15 min. Labeled cells were washed once with Macs buffer, resuspended in 2 mL of Macs buffer, and separated by magnetic column. Recovered CD4$^+$ and CD8$^+$ were determined to be >95% positive. Purified CD3$^+$(2 10^6 cells/mL per well), CD4$^+$ (2×10^6 cells/mL per well), and CD8$^+$(1×10^6 cells/mL per well) T-cells were resuspended in DMEM complete medium supplemented with 10% FBS, 0.005% (v/v) 2-mercaptoethanol (Gibco™, Invitrogen, Grand Island, NY), and antibiotics (100 μg/mL penicillin G and 50 μg/mL gentamycin sulfate) and subsequently overlaid onto prepared macrophages. Macrophage presenters were previously infected with BCG (1:1), BCG (10:1), or remained non-infected, and either treated with or without lactoferrin (100 μg/mL) for 72 h. Prior to co-culture with isolated T-cells, macrophage presenters were thoroughly washed with 1× PBS. Supernatants were collected at 72 h and cytokines analyzed by ELISA. For intracellular staining, splenocytes were restimulated with ConA (2 μg/mL) or PMA (10 ng/mL) and ionomycin (250 ng/mL) in the presence of 1 μL/well BD Golgi Plug (BD Biosciences, San Diego, CA) for 6 h and isolated for FACS analysis.

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2.5. Examination of BCG proliferation

Effect of lactoferrin on BCG proliferation was performed for growth in broth culture or within macrophages. For broth culture, BCG, at log phase growth, was seeded (10^5 CFU/mL) into Dubos base with 10% supplement with increasing concentrations of bovine lactoferrin (0, 100, or 1000 μg/mL). For growth within cells, bone marrow derived macrophages (5×10^5 cells/mL per well) were infected with BCG at MOI 1:1 or 10:1, with or without increasing concentrations of lactoferrin (1, 10, or 100 μg/mL). At days 1, 3, and 5 post-infection, cells were lysed with 500 μL/well of 0.05% SDS, incubated at 37 °C, and then neutralized with an equal volume of 15% BSA. Aliquots of broth culture or cell lysates were serially diluted in 1× PBS and 100 μL plated onto 7H11 agar plates and incubated at 37 °C. CFU were enumerated as described above.

2.6. FACS analysis

Antibodies (1 μg/10^6 cells per 50 μL) in staining buffer (1% BSA in 1× PBS) were added to isolated cells following treatment on ice with Fc Block™ (CD16/32, BD Biosciences Pharmingen, San Diego, CA). Macrophages were incubated with anti-mouse F4/80-FITC (Cell Sciences), CD11c-PE, I-A^b-FITC, H-$2k^b$-FITC, CD80-PE, CD86-PE, or CD40-PE (BD Biosciences Pharmingen) on ice for 30 min. Macrophages were washed with staining buffer and fixed with 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ). For intracellular staining, splenocytes were block with Fc Block™ and stained with CD4-FITC or CD8-FITC on ice for 30 min. Intracellular staining was conducted after the cells were fixed with 4% paraformaldehyde on ice for 15 min and made permeable with BD Perm/Wash solution (BD Biosciences Pharmingen, San Diego, CA). Cells were further stained for IFN-γ-PE on ice for 1 h. Flow analysis was conducted using Coulter FlowCentre™ (EPICS XL-MCL). Graphs were generated with WinMDI 2.8 or GraphPad Prizm 4.

2.7. ELISA (enzyme linked immuno-sorbant assay)

Supernatants were assayed for cytokine production using the DuoSet ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Supernatants were assayed for production of T-cell cytokines (IFN-γ, IL-2, and IL-4), proinflammatory mediators (TNF-α and IL-6), and T-cell mediators (IL-12p40 and IL-10). The lower limits of assay detection for all cytokines were between 15 and 32 pg/mL.

2.8. Statistics

All experiments were repeated, at least, in triplicate. CFU were enumerated from triplicate platings. ELISA analysis was averaged from triplicate wells. Changes in surface expression of CD11c, I-A^b, H-$2k^b$, CD80, CD86, or CD40 were compared across repeated experiments with significance determined by paired *t*-test. Statistical analysis was carried out using one-way ANOVA, and differences were considered significant at $p < 0.05$.

3. Results

3.1. Lactoferrin increases of MHC II expression on BCG-infected macrophages

To address whether lactoferrin could mediate expression of presentation molecules on antigen presenting cells, BCG-infected bone marrow derived macrophages (BMMs) were examined for expression of MHC I (H-$2k^b$) and MHC II (I-A^b) and co-stimulatory molecules (CD80, CD86, and CD40) when cultured with or without lactoferrin. Bone marrow derived macrophages remained non-infected or infected with BCG (MOI 10:1) in the presence or absence of lactoferrin (100 μg/mL). Expression of MHC II was low in non-activated BMMs (range for four experiments, 4.0–6.8%). Lactoferrin had only a slight, non-significant effect on non-activated BMM expression of MHC II (4.0–7.5%). Expression of MHC II on BCG-

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infected BMMs increased in the presence of lactoferrin (Fig. 1, top). Statistical analysis of results from four experiments found a modest but significant ($p < 0.05$) increase of MHC II observed on BCG-infected BMMs that were cultured with lactoferrin. These results correlate well with previously published observations of MHC II expression on macrophages differentiated using an alternative method [41]. Lactoferrin had no effect on relative surface expression of MHC I, CD80, CD86, or CD40 on either BCG-infected or non-infected BMMs (data not shown).

3.2. Lactoferrin reverses BCG-mediated suppression of MHC II expression in BMMs

Naïve (non-activated) BMMs express only low levels of presentation and co-stimulatory molecules unless activated. To study effects of lactoferrin on activated macrophages, experiments were repeated in the presence of IFN-γ. BMMs were cultured with or without BCG (MOI 10:1), with or without lactoferrin (100 μg/mL), and with recombinant IFN-γ (10 ng/mL). Cells were analyzed for expression of MHC I, MHC II, CD80, CD86, and CD40 after 72 h. The IFN-γ activated BMMs expressed high levels of MHC II (>80%) (Fig. 1, bottom); addition of lactoferrin had no observable effect on MHC II expression on these cells. BCG infection alone caused a dramatic decrease of MHC II^{+} expressing cells ($p < 0.001$) under IFN-γ stimulating conditions. The presence of lactoferrin during BCG infection allowed a significant proportion of BMMs to retain their expression of MHC II ($p < 0.01$). While the lactoferrin treated BCG-infected BMMs did not fully recover the level of expression found in IFN-γ stimulated uninfected BMMs, lactoferrin was clearly able to modulate the extent of BCG mediated MHC II down regulation.

An additional experiment was performed to evaluate events where stimulation of macrophages occurs post treatment of infection with lactoferrin. Macrophages were initially exposed to BCG and lactoferrin for 3 days. Cells were washed to remove extracellular BCG and lactoferrin, and then restimulated with 10 ng/mL of mouse recombinant IFN-γ. In this case, non-infected BMMs cultured with lactoferrin significantly ($p = 0.034$) increased the number of BMMs positive for MHC II expression compared to the non-treated cells (Fig. 2A). However, addition of IFN-γ to the lactoferrin treated BCG-infected BMMs did not induce significant change in expression of MHC II compared to the BCG alone infected BMMs. No significant changes were observed with expression of MHC I in all groups examined (data not shown).

3.3. Macrophages pre-exposed to BCG and lactoferrin enhance the CD86/80 ratio in response to IFN-γ stimulation

The expression of co-stimulatory molecules CD80 and CD86 on IFN-γ activated BMMs was also found to be differentially modulated by lactoferrin. There was a modest, but non-significant, trend of decrease in CD80 expression observed in non-infected BMM macrophages pre-treated with lactoferrin (range for 4 experiments, 32–49%) compared to those not treated with lactoferrin (36–63%). However, there was a significant ($p < 0.05$) decrease in CD80 expression on BCG-infected BMM pre-treated with lactoferrin (Fig. 2B).

Changes were also observed in CD86 expression which were unique from those seen for CD80 (Fig. 2C). In this case, there was a significant ($p < 0.01$) decrease in CD86 expression observed in non-infected macrophage pre-treated with lactoferrin (range for 4 experiments, 9–20%) compared to those not treated with lactoferrin (19–35%). However, there was no significant difference observed in CD86 expression from IFN-γ activated BCG-infected BMMs pre-treated with BCG/lactoferrin (17–28%) compared to BMMs infected with BCG only (23–25%). The relative ratio of CD86 to CD80 expression was further compared. BMM pre-treated with lactoferrin and infected with BCG showed a significant ($p < 0.005$) increase in the CD86:CD80 ratio (range for three experiments 0.65–0.93) when compared to non-infected lactoferrin pre-treated BMMs (0.31–0.55).

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3.4. Increased IFN-γ production from BCG sensitized CD3$^+$ splenocytes stimulated with lactoferrin treated presenters

The identified changes in MHC Class II expression and levels of CD80 and CD86 on the surface of infected cells indicate that lactoferrin may be capable of enhancing antigen presentation by BCG-infected BMMs. To examine this hypothesis, CD3$^+$, CD4$^+$, or CD8$^+$ splenocytes were recovered from BCG immunized mice, and incubated with BMM presenting cells that were BCG-infected in the presence or absence of lactoferrin (100 μg/mL).

There was a modest, but significant ($p < 0.001$), increase in production of IFN-γ from CD3$^+$ splenocytes overlaid onto BCG-infected BMMs treated with lactoferrin (Fig. 3). The sensitized CD3$^+$ cells produced 116.0 ± 10.4 pg/mL of IFN-γ when lactoferrin was added, compared to only 59.3 ± 12.5 pg/mL in the non-lactoferrin treated group. When examined for response on highly infected presenting cells (MOI 10:1), the response remained significant, with 723.7 ± 9.3 pg/mL of 116.0 ± 10.4 pg/mL IFN-γ produced in combination with the lactoferrin treatment, compared to 656.3 ± 16.6 pg/mL with no lactoferrin added. The subpopulation of responding CD4$^+$ lymphocytes was further examined by intracellular flow cytometric analysis (Fig 4). BCG-infected macrophages cultured with lactoferrin significantly increased the mean fluorescent intensity (MFI) of CD4$^+$IFN-γ$^+$ cells compared to macrophages infected with BCG only. There were no differences observed in the number of CD4$^+$ IFN-γ$^+$ cells stimulated by BCG-infected macrophages treated with or without lactoferrin. This suggests that macrophages infected with BCG in the presence of lactoferrin were able to increase IFN-g production from CD4$^+$ splenocytes.

No significant differences were observed in production of IL-12, a macrophage cytokine that can mediate production of IFN-γ (Table 1). Analysis of proinflammatory cytokines, TNF-α and IL-6 production from CD3$^+$ splenocytes incubated onto BCG-infected (MOI 10:1) macrophages in the presence of lactoferrin were significantly elevated compared to the non-lactoferrin group. Since both macrophages and CD3$^+$ splenocytes are capable of producing TNF-α and IL-6, CD3$^+$ splenocytes were further examined when incubated with similarly treated paraformaldehyde fixed macrophages. Similar increases in production of TNF-α (135 ± 9 pg/mL without lactoferrin vs. 239 ± 9 pg/mL with lactoferrin; $p < 0.001$) and IL-6 (17 ± 1 pg/mL without lactoferrin vs. 43 ± 5 pg/mL with lactoferrin; $p < 0.001$) were observed.

3.5. No effect of lactoferrin on intracellular proliferation of BCG

The increased ability of lactoferrin treated BCG-infected BMMs to stimulate IFN-γ production from sensitized T-cells suggested greater proficiency in presenting BCG antigens. However, antigen loading onto MHC II involves active processing of the intracellular bacteria which may be directly explained by changes in BCG proliferation. Therefore, the effect of lactoferrin on BCG proliferation within BMMs was examined. BMMs were infected with BCG with or without lactoferrin (100 μg/mL), and with or without stimulation of IFN-γ. No differences were observed in BCG intracellular proliferation in BMMs treated with or without lactoferrin (Fig. 5A). Similarly, in BCG-infected BMMs activated with exogenous IFN-γ, no differences were observed between the lactoferrin and non-lactoferrin groups (Fig. 5B). Additionally, lactoferrin was examined for potential of direct anti-mycobacterial activity. No differences were observed in BCG proliferation when grown in culture with or without lactoferrin (Fig. 5C).

4. Discussion

The continuously falling efficacy of the BCG vaccine has generated intense research into development of novel vaccines to fight against TB, a disease that affects a third of the world's populations [1-4]. While examinations of newly developed attenuated virulent MTB and recombinant BCG strains have shown comparable efficacy with the current BCG vaccine, both

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involve use of live bacteria, with no comparative history of clinical safety [42-46]. The current BCG vaccine is the most widely administered vaccine in the world, and has a long established history of known benefits and risks [47]. Thus, the quickest and most likely route to improving TB vaccine for human use is through improvement of the current BCG. This strategy has already produced a vaccination regimen that is undergoing Phase II clinical trials by using an adenovirus containing Ag85 as a booster to the BCG vaccine [45].

The potential for use of naturally occurring lactoferrin as an adjuvant to augment immune function has been validated with *in vivo* studies that showed no toxic effects attributed to oral delivery of lactoferrin [48-50]. There are no published reports directly examining toxicity of injected bovine lactoferrin when utilized as an adjuvant, however, high concentrations (5–10 mg/mouse) of bovine lactoferrin are often delivered in experiments examining LPS and bacterial insults in murine models of sepsis [51-54] with no overt or reported toxicity. Previous studies demonstrated that addition of lactoferrin to the BCG vaccine increased host protection against subsequent virulent MTB challenge as observed by a decrease in organ bacterial load and a reduction in pulmonary disease pathology. This improvement in host response to disease correlated to an increase in development of a protective mycobacterial antigen-specific T-cell response, specially a T_H1 response hallmarked by production of IFN-γ [22,23]. Thus, lactoferrin has the ability to modulate innate immune function during vaccination in a way that augments generation of long-lasting protective immunity.

It has long been recognized that lactoferrin exerts effects on a variety of leukocytes, including macrophages [55-57], the main host cell for both BCG and MTB [58,59]. Previous studies demonstrated that lactoferrin is capable of enhancing BCG-infected macrophage production of IL-12:IL-10 ratio [34], thus generating a cytokine environment that is favorable for promotion of T_H1 immunity [24-26]. It was hypothesized that lactoferrin also enhanced efficacy of the BCG vaccine *in vivo*, in part, by modulating the ability of macrophages to present antigen and for subsequent stimulation of T-cells.

In this study, lactoferrin caused an increase in MHC II expression on BCG-infected bone marrow derived macrophages, suggesting an increase in the ability of infected cells to present antigen to lymphocytes. Resting BMMs express very low levels of MHC II on their surface, therefore, the effect of lactoferrin was also examined on IFN-γ activated BCG-infected BMMs. BCG infection of IFN-γ activated BMMs dramatically decreased MHC II^+ BMMs, a trend that has been previously reported [60-62]. The presence of lactoferrin was able to reverse a significant percentage of the BCG induced down regulation of MHC II surface expression, again suggesting that lactoferrin enables activated macrophages to retain their ability to present antigen to T-cells. In the larger context of the diminished historical efficacy of the BCG vaccine, this ability of BCG to decrease MHC II expression in activated macrophages is hypothesized to be a natural immune evasion mechanism that limits development of an adequate immune response [63,64]. Lactoferrin is capable, in part, of reversing this inhibition.

In addition to examining the effect of lactoferrin on resting and stimulated BMMs, lactoferrin was also examined for its ability to modulate BMMs' responsiveness to exogenous IFN-γ. In the series of events that occur *in vivo* during vaccination, there is a lag time between when macrophages encounter BCG and lactoferrin, and the subsequent recruitment and activation of T-cells that would result in IFN-γ production to augment host intracellular events to control infection. The effect of BCG to downregulate expression of MHC II is indicative that the vaccine strain retains immune evasion mechanisms that are observed in the virulent MTB strains. Another hallmark of mycobacteria evasion mechanism involves attenuation of macrophage upregulation of MHC II in response to IFN-γ stimulation [65,66]. While lactoferrin can act synergistically with IFN-γ at the time of BCG infection, there were no changes in MHC II expression when macrophages were stimulated with IFN-γ 72 h after BCG

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infection. This strongly suggests a need for lactoferrin to be present at specific times relative to antigen delivery during the macrophage activation process. Although there was no change in MHC II in macrophage response to IFN-γ after previous exposure to BCG, these results indicate that lactoferrin is able to modulate response in a way to maximize T-cell stimulation, possibly through increasing the relative ratios of surface CD86 to CD80 expressed on cultured BCG-infected macrophages.

Both CD86 and CD80 are considered necessary co-stimulatory molecules for T-cell activation, functioning as secondary components involved in antigen presentation by MHC molecules. Significant disagreement exists in the literature on the possible differential functions of CD86 and CD80 [67,68]. Recently, support is given to indicate that they may preferentially bind different ligands on T-cells; CD86 may preferentially bind CD28, the ligand for T-cell activation, and CD80 may preferentially bind CTLA-4, a ligand to elicit T-cell anergy [69-71]. While lactoferrin cultured BCG-infected BMMs exhibit a total reduction in both molecules, there was a consistent increase of relative CD86:CD80 ratio in response to IFN-γ, suggesting that these macrophage populations may have a better potential to engage the CD28 ligand for activation of T-cells.

The effect of lactoferrin on BCG-infected BMMs suggested that it would increase the ability of macrophages to stimulate T-cells. Events relating to mycobacterial-specific antigen presentation were examined. BCG sensitized splenocytes were incubated with BCG-infected BMMs, cultured with or without lactoferrin. The lactoferrin treatment of BCG-infected BMMs enhanced production of IFN-γ from $CD3^+$ splenocytes. In addition, $CD4^+$ T-cells were shown to increase the amount of IFN-γ per cell in response to presented BCG antigens. Production of IL-12, which can indirectly influence IFN-g production [26,27,72], was not affected, suggesting that the increase in T-cell IFN-γ production was the result of direct cell to cell contact instead of promotion by the cytokine environment. Overall, the increase in T-cell IFN-γ production suggests that lactoferrin cultured BCG-infected BMMs can generate signals via surface molecules and cytokine expression which would aid in the development of *in vivo* T_H1 immunity observed in previous studies [22,23].

Lactoferrin was originally defined as an iron binding protein that directly affects bacteria, functionally recognized as both bacteriostatic and bacteriocidal [11-13,73]. However, there was no direct bacteriostatic or bacteriocidal effect of lactoferrin on BCG proliferation. In addition, we also examined the effect of lactoferrin on macrophage intracellular control of BCG proliferation. One main mechanism for macrophage control of intracellular BCG involves production of NO [74,75], which can be stimulated by activation with IFN-γ[76]. Also, lactoferrin alone has been shown to increase macrophage NO production [77]. However, we observed no effect of lactoferrin on intracellular proliferation of BCG. Therefore, the enhanced antigenic stimulation of T-cells does not suggest an increase or mediation in intracellular processing mechanisms, which would result in more efficient antigen presentation. Indeed the studies presented here suggest that the effect of lactoferrin is through mechanisms other than mediation of macrophage direct intracellular killing events.

Receptors for lactoferrin have been identified on a variety of leukocytes, including macrophages [78]. A role for involvement utilizing c-type lectin receptors has also been identified; lactoferrin is able to prevent uptake of HIV by binding to DC-SIGN on human dendritic cells [79], and the effect of lactoferrin to enhance the DTH response *in vivo* can be blocked by addition of mannose [20]. Macrophage mannose receptor and the murine homolog of DC-SIGN (mSIGN-R1) are involved in binding and/or uptake of BCG and MTB [80-83], therefore addition of lactoferrin may influence this initial infection event. We have previously observed that BCG uptake is not hindered by the presence of lactoferrin [41]. We conclude that the effect of lactoferrin in the studies reported here demonstrates direct modulation of

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macrophage responses and not indirect interference of mechanical events regulating uptake of BCG. However, it is clear that there needs to be an awareness in trying to generalize immune modulatory potential of the lactoferrin molecule, as glycosylation pattern may be different depending on phenotypic cell source. Indeed, different glycoforms of human recombinant lactoferrin (sialylated and non-sialylated) expressed statistically different effects on *in vitro* secondary humoral immune responses [84].

These results demonstrate lactoferrin as a molecule capable of modulating macrophages to promote T-cell stimulation through upregulation of presentation and co-stimulatory molecules. Clearly macrophages play an important role in uptake, containment and control of BCG and MTB. This study is the first step in the examination of the mechanisms underlying lactoferrin effects as an adjuvant component. Efforts are now underway to extend this assessment to determine the action on dendritic cell populations, as those cells are critical in directing immune function of naïve lymphocyte populations [85] and for developing host immune responses to control MTB infection [86]. Overall, lactoferrin may present an important adjuvant to enhance efficacy of not only the BCG vaccine, but also other vaccines with poor immunogenic abilities to promote antigen-specific, cell mediated immunity.

Acknowledgements

The research was presented in part at the 8th International Conference on Lactoferrin, held in October, 2007, in Nice, France. The work was accomplished with support from NIH grants 1R41GM079810-01 and R42-AI051050-02.

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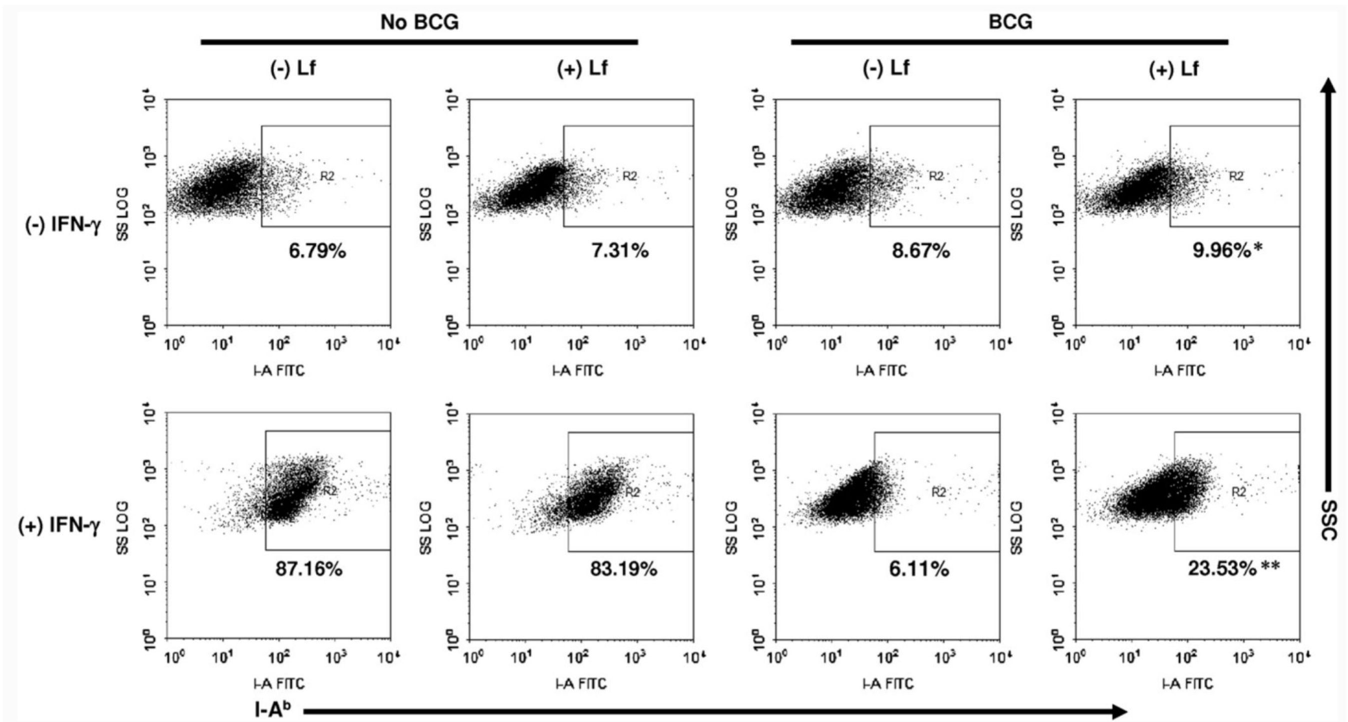

Fig. 1.

Lactoferrin mediated change in surface expression of I-A^b (MHC II) in BCG-infected macrophages. Naïve bone marrow derived macrophages (BMMs), or BCG-infected BMMS (MOI 10:1) were cultured with or without lactoferrin (100 μg/mL), and stained for surface expression of I-A^b-(MHC II) after 72 h. Cells were cultured either alone (top) or stimulated with IFN-γ (10 ng/mL) (bottom). Positive events were gated against the isotype control; representative findings from duplicate readings from 4 repeated experiments were analyzed by paired *t*-test. *$p < 0.05$; **$p < 0.001$ relative to non-lactoferrin treated groups.

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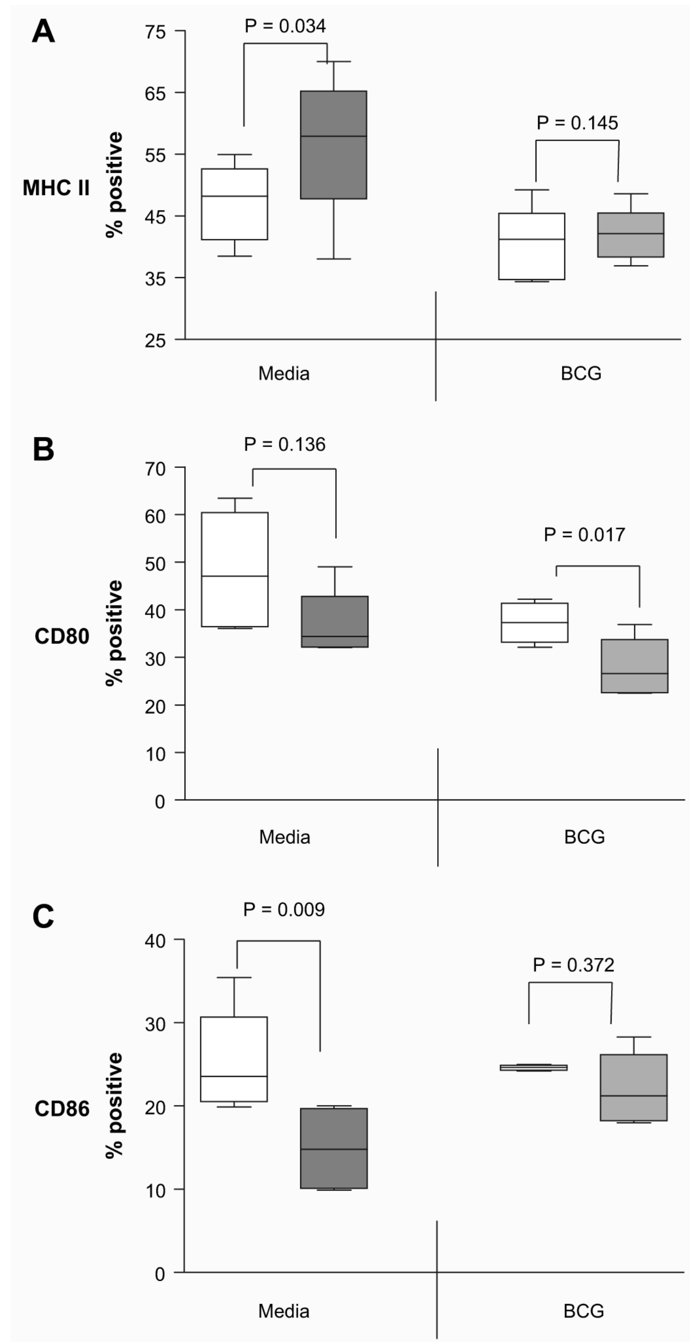

Fig. 2.

Effect of lactoferrin on presentation molecules expression on IFN-γ stimulated BCG-infected macrophages. Non-infected or BCG-infected BMMs were cultured with (shaded bars) or without lactoferrin (open bars) (100 μg/mL), and stimulated with 10 ng/mL mouse recombinant IFN-γ. After 72 h, cells were stained for surface expression of (A) I-A^b-(MHC II), (B) CD80, or (C) CD86. Positive events were gated against isotype controls. Percent positive cells from 4 or more repeat experiments were analyzed by paired *t*-test, with *p* value indicated.

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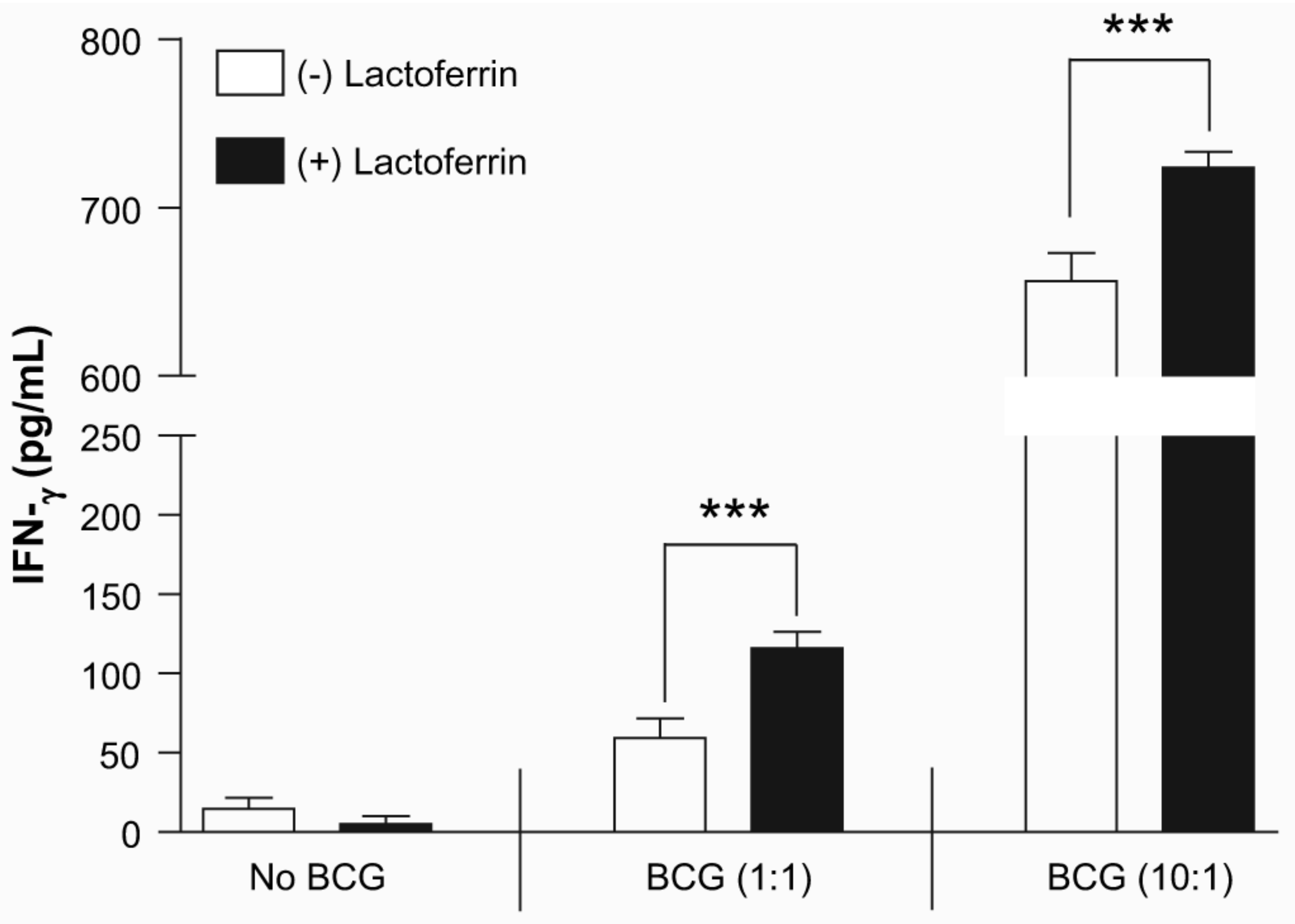

Fig. 3.

Elevated IFN-γ production from sensitized T-cells incubated with lactoferrin treated antigen presenting cells. $CD3^+$ splenocytes isolated from mice previously immunized with BCG were incubated with BCG-infected bone marrow derived macrophages (MOI 1:1 or 10:1) in the presence or absence of lactoferrin (100 μg/mL). Supernatants were collected at 72 h and analyzed by ELISA for IFN-γ production. ***$p < 0.001$.

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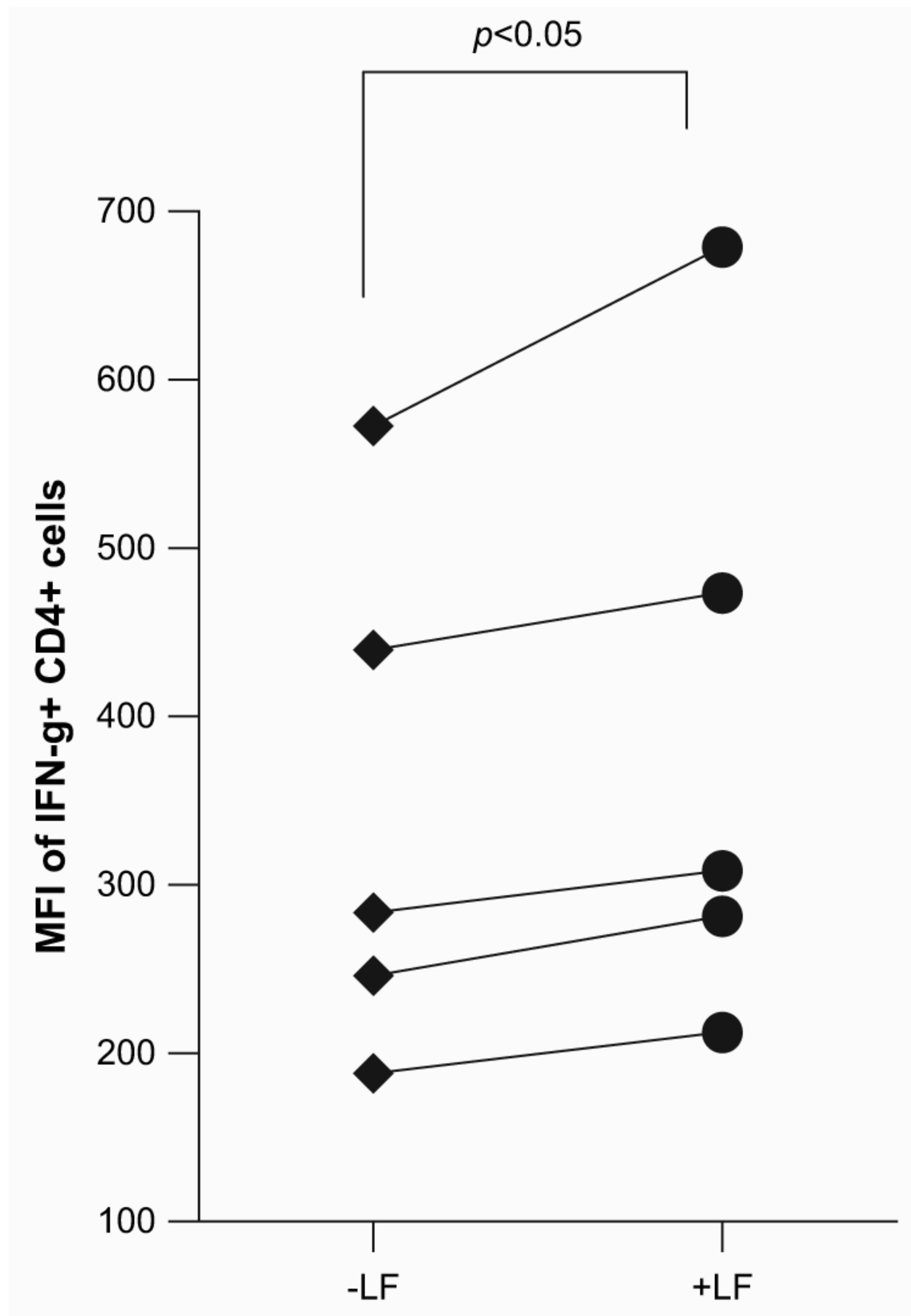

Fig. 4.

Lactoferrin enhanced BCG-infected macrophages stimulation of IFN-γ production from $CD4^+$ splenocytes. $CD3^+$ or $CD4^+$ splenocytes were isolated from mice previously immunized with BCG. Bone marrow derived macrophages were infected with BCG in the presence or absence of lactoferrin (100 μg/mL) for 72 h prior to T-cells co-culturing. ConA (2 μg/mL) or PMA/ionomycin (10/250 ng/mL) restimulated cells were isolated and stained for CD4-FITC and IFNγ-PE. Matched samples are shown splenocytes from 5 individual mice, reflecting BMMs incubated with or without lactoferrin.

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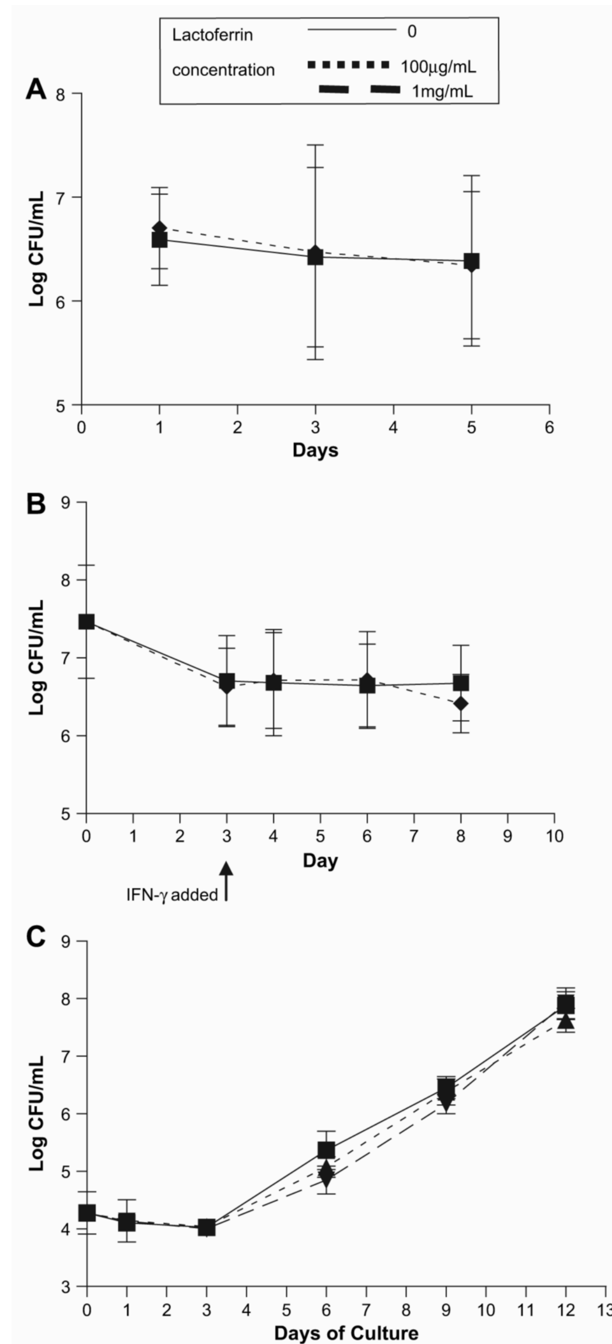

Fig. 5.

Lactoferrin does not affect extracellular or intracellular proliferation of BCG. The effect of lactoferrin growth on BCG was evaluated. (A) BMMs were infected with BCG with or without lactoferrin (100 μg/mL). (B) BMMs were infected with BCG with or without lactoferrin (100 μg/mL) for 72 h, and stimulated with IFN-μ (10 ng/mL). (C) BCG was grown on an orbital shaker in Dubos broth base with or without lactoferrin (100 μg/mL, 1 mg/mL). Serial dilutions of cell lysates or broth culture were plated onto 7H11 plates at indicated days post culture, and CFU were enumerated.

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Table 1

Lactoferrin mediation of pro-inflammatory cytokine production from sensitizedCD3+ cells cultured with BCG-infected macrophages

	No BCG	BCG (1:1)	BCG (10:1)
IL-12(p40)			
(−) Lf	6 (5)	51 (5)	1176 (117)
(+) Lf	5 (5)	48 (12)	1089 (43)
TNF-α			
(−) Lf	65 (55)	45 (4)	264 (26)
(+) Lf	58 (19)	55 (6)	445 (44)*
IL-6			
(−) Lf	17 (7)	11 (9)	91 (5)
(+) Lf	7 (7)	17 (2)	148 (3)*

$CD3^+$ splenocytes isolated from mice previously immunized with BCG were incubated with non-infected bone marrow derived macrophages, or with BCG-infected BMM (MOI 1:1 or 10:1), in the presence or absence of lactoferrin (100 μg/mL). Secreted IL-12p40, TNF-α and IL-6 were and analyzed by ELISA. Average values of triplicate wells are given in pg/ml (with standard deviation)

* $p < 0.001$.